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AgNORs and Their Relationship to Cell Size, Histological Grade, Lymph Node Involvement, Metastases, and Survival Pattern in Carcinoma of the Breast: A Study From South India

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An AgNOR count using the Smith and Crocker [Histopathology 12:113–125, 1988] method of staining was performed on 200 cases of carcinoma of the breast. A count of coarse AgNORs per nucleus was made on 50 random cells and the mean of their number per nucleus calculated. The relationship of a single variable "AgNOR count" to other variables such as cell size, histological grade, number of positive ipsilateral axillary lymph nodes, and presence of metastasis in regions other than the ipsilateral axillary lymph nodes was found using a univariate method of analysis. Also, the effect of different independent variables, e.g., number of AgNORs, cell size, histological grade, number of positive axillary lymph nodes, and metastasis on a single variable, i.e., 4-year period of survival, was also assessed by a univariate method of statistical analysis. It was found that the AgNOR count was significantly related to the cell size, histological grade, and presence of metastasis. Large cells, grade III tumors, and neoplasms with evidence of metastasis showed larger numbers of AgNORs in their nuclei. It was observed that the number of AgNORs significantly affected the 4-year survival of patients. The higher the AgNOR counts, the poorer were the chances of surviving for 4 years. The other factors that influenced survival in the present study were the number of positive axillary lymph nodes and metastasis to sites other than axillary lymph nodes.

KEY WORDS: **breast, carcinoma, AgNORs, cell size, histological grade, survival**

INTRODUCTION

Nucleolar organizer regions (NORs) are loops of ribosomal DNA that occur in nuclei and direct ribosome and protein formation. NORs possess ribosomal RNA genes, which are transcribed by RNA polymerase-1 and ultimately direct ribosome and protein synthesis [1,2].

For several years, a silver-staining technique on paraffin sections has made it possible to visualize these NORs as brown-black dots in the nuclei of cells by virtue of the argyrophilia of the NOR-associated proteins. It has been suggested that the number of NORs in nuclei may reflect the state of cellular activity and thus the malignant potential of these cells [2,3].

The present study aims at visualizing these argyrophilic nucleolar organizer regions (AgNORs) in breast carcinoma patients and relating their numbers per nucleus

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to the size of the nucleus, the histological grade of the neoplasm, number of positive axillary lymph nodes, presence of metastases, and survival of the patient.

MATERIALS AND METHODS

The material and data for this study were taken from the Archives of the Department of Pathology (St. John's Medical College, Bangalore), which serves ~15 million persons in the four southern states of India, namely, Karnataka, Tamil Nadu, Andhra Pradesh, and Kerala. Included were specimens of modified radical mastectomies with axillary lymph node clearance. Specimens where lymph node dissections were not performed and small biopsies on the breast were excluded from this study.

Procedure

A routine Hematoxylin and Eosin stain was used to view the exact histomorphology of the lesion. Depending on the nuclear size, all neoplasms were divided into three categories: large cell, medium cell, and small cell. A neoplasm was considered to be large cell in type if 75% of its cells or more showed a nuclear diameter of 12 μm or more. A cell was considered to belong to the medium cell type if 75% of its cells or more showed a nuclear diameter of 8–11 μm, and a neoplasm was considered to be small cell in type if the nuclear diameter was 5–7 μm in size in 75% or more of its neoplastic cells. The cell size was categorized irrespective of the histologic type of tumor, i.e., specific or not otherwise specified (NOS).

A histological grading of the neoplasm was carried out using the Bloom and Richardson [4] grading based on tubule formation, nuclear morphology, and mitotic activity. A score of 3, 4, or 5 was considered as grade I, 6 and 7 as Grade II, and 8 or 9 as Grade III.

The number of axillary lymph nodes showing metastasis was recorded. Cases were subsequently categorized as those showing 1–4 or >4 lymph node involvement.

NOR staining was done on routinely processed formalin-fixed paraffin sections using the Smith and Crocker [5] method. Coarse and fine AgNORs were observed. Only the coarse AgNORs were used for NOR assessment and the fine ones ignored. Fifty cells were randomly assessed for their AgNOR content using the 100x oil immersion lens and a mean of the AgNOR number calculated per nucleus by two observers. The average of the two observations was taken into account.

Where metastases occurred, either at the time of the first or subsequent examinations, the site of metastasis was noted. This was detected by either radiologic technique, biopsy, or a CT scan.

A follow-up of the patients was done with the help of facilities available at the Indian Council of Medical Research (ICMR) Registry at Bangalore. When death occurred, the total duration of patient survival was noted.

Statistical Analysis

A univariate procedure was used: (1) to compare the interrelationship of a single variable, AgNORs, with other variables, i.e., cell size, histological grade, number of positive axillary lymph nodes, and presence of metastases, and (2) to find out the effect of the different independent variables, e.g., cell size, histological grade, AgNORs, number of positive axillary lymph nodes, and metastasis on a single dependent variable, e.g., survival or non-survival at 4 years follow-up. Patients who had not been followed up for the adequate period of 4 years postsurgery were not taken into consideration and hence disregarded in the survival analysis.

The data were analyzed as follows: For *qualitative data,* to compare frequencies across groups, the Chi-square and Fisher's tests were used. The Chi-square test was used with Yates' continuity correction in all 2×2 contingency tables. In large contingency tables (e.g., 3×4), when expected frequencies in groups of cells were small, the tables were condensed based upon a prior assumption to meet the requirements of Chi-square testing. The Fisher's exact (two-tail) probability test was used in all 2×2 and 2×3 contingency tables wherein >20% of cells had an expected frequency <5.

For *quantitative* data, to compare means +/− standard deviations across groups, the independent sample (Student's) *t*-test was used where variances of the groups were comparable, and the *t*-test with modified degrees of freedom was used where variances of the groups significantly differed. (The F ratio of variances was used to examine homogeneity of variances.)

For *determination of correlations,* the Pearson's product-moment correlation coefficient was employed.

RESULTS

Of 86,000 surgical biopsies recorded over a period of 26 years, from April 1, 1966 to March 31, 1992, at the Archives of this department, 5,500 were breast biopsies, 445 of which were diagnosed as carcinomas. Of these 445 patients, 200 had modified radical mastectomies over this period and were included in this study.

Cell Size

Based on the nuclear diameter on the H & E stained sections, 110 cases belonged to the large cell type, 58 to the medium cell category, and 32 to the small cell category.

Histological Grade

On an extended Bloom and Richardson [5] method of grading, 175 carcinomas belonged to grade III, 24 tumors belonged to grade II, and only one neoplasm was graded as I.

TABLE I. Carcinoma of the Breast: Relationship Between AgNORs and Cell Type

AgNORs/nucleus	1	2	3	4	5	6
Cell type:						
Large cell	2	5	40	44	17	2
Medium cell	0	28	28	2	0	0
Small cell	5	19	8	0	0	0

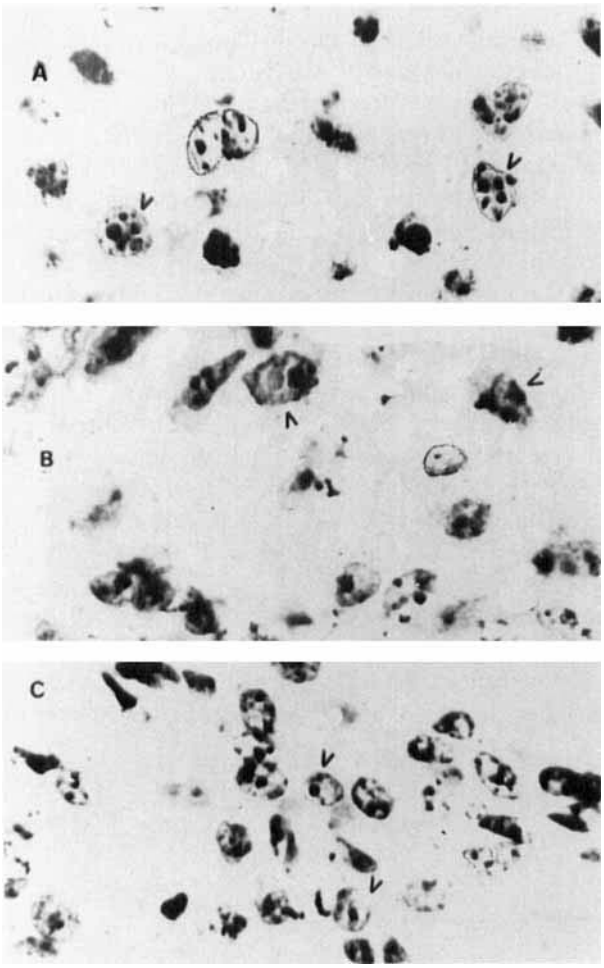

Fig. 1. **A.** Large cells (nuclear diameter of 12 μm or more) with ~5–6 AgNORs per nucleus (see arrows). **B.** Medium cells (nuclear diameter 8–11 μm) with 2 AgNORs per nucleus (see arrows). **C.** Small cells (nuclear diameter 5–7 μm) with one AgNOR per nucleus (see arrows). AgNOR stain × 1000 magnification.

AgNORs

The number of AgNORs varied from 1–6 in each nucleus.

AgNORs and cell type. Table I shows the relationship between the AgNORs and the cell type. More coarse AgNORs (4–5/nucleus) were seen in large cell carcinomas, whereas in medium cells, most cases showed a maximum number of 2 or 3 AgNORs per nucleus, and in small cell carcinomas even less than this (1–2 AgNORs/nucleus) (Fig. 1).

TABLE II. Carcinoma of the Breast: Relationship Between AgNORs and Histological Grade

	Grade		
AgNORs/nucleus	I	II	III
1	1[a]	0	6
2	0	16	36
3	0	7	69
4	0	1	45
5	0	0	17
6	0	0	2

[a]In the Chi-square computations undertaken for the data presented in Table II, the grade I data were ignored because only one of the 200 cases fell into this group.

TABLE III. Carcinoma of the Breast: Relationship Between AgNORs and Axillary Lymph Node Involvement

AgNORs/nucleus	No lymph node involvement	1–4 lymph nodes involved	>4 lymph nodes involved
1	2	5	0
2	22	28	2
3	29	39	8
4	14	27	5
5	8	8	1
6	0	0	2

AgNORs and histological grade. Most grade III tumors showed 3–4 AgNORs per nucleus. Most cases with a histological grade of II showed two AgNORs per nucleus. The only one case of grade I showed a single AgNOR per nucleus (Table II).

AgNORs and lymph node involvement. Axillary lymph nodes positive for metastasis were present in 125 of the 200 cases. In 107 of the 125 cases, 1–4 lymph nodes were involved, and in 18 cases >4 lymph nodes were involved. Neoplasms with axillary lymph node involvement had chiefly 2, 3, or 4 AgNORs per nucleus (Table III).

AgNORs and metastasis (other than axillary lymph nodes). Apart from axillary lymph nodes, spread to other sites was seen in 62 cases and was as follows: operated scar—9, liver—20, lung—17, ovaries—14, bone—6, abdomen including peritoneum—5. It was observed that five cases of lung metastasis were also the same ones showing liver metastasis; two of the cases with bone metastasis also showed spread to the lung and two others to the ovaries.

TABLE IV. Carcinoma of the Breast: Relationship of AgNORs and Metastasis

AgNORs/nucleus	Number of cases showing metastasis
1	2
2	9
3	27
4	15
5	8
6	1

Most cases with metastasis showed 3–4 AgNORs per nucleus (Table IV).

Follow-Up

A follow-up of the 200 cases was done up to a period of 4 years postsurgery. Sixty-nine patients survived for 4 years or longer; 21 patients died within this period. In the remainder of the sample, the patients had not been followed up for an adequate period of time to allow classification as alive or dead at 4 years postsurgery.

Statistical Data

Univariate analysis of AgNORs and other variables. The results of the Chi-square analysis done on AgNORs and other variables are: (1) AgNORs and cell type—Chi square = 90.71958; $df = 2$; $P < 0.0001$; (2) AgNORs and histological grade—Chi square = 19.27; $df = 3$; $P < 0.0001$; (3) AgNORs and lymph nodal involvement—Chi square = 4.37; $df = 4$; p = NS (Not Significant); (4) AgNORs and metastasis—Chi square = 44.8387; $df = 5$; $P < 0.0001$.

Univariate analysis of survival patterns. A univariate assessment was made of the variables influencing the 4-year survival. Only those patients followed up for a period of 4 years were included in the study. The results of the analysis are shown in Table V. It was observed that survival was significantly related to the AgNOR number per nucleus, number of positive axillary lymph nodes, and metastases.

DISCUSSION

It is a well-established fact that the number of nucleolar organizer regions and rate of cellular activity are closely related. These nucleolar organizer regions are readily demonstrated by means of the argyrophilia of their associated proteins. The method of Smith and Crocker [5] is easy to perform, the only disadvantage being the heavy precipitation of silver on the section obscuring easy viewing.

Smith and Crocker [5] found that the total numbers of AgNORs in malignant breast lesions significantly exceeded those in the normal breast or even in benign breast lesions. These authors have also stated that such AgNOR counts particularly helped to distinguish benign states like sclerosing adenosis from invasive carcinomas.

AgNOR studies also have been done on lymphomas by Crocker and Nar in 1987 [2], and the results have shown that high grade lymphomas with a high rate of cell turnover contained multiple AgNOR dots in contrast to low grade tumors, which possessed approximately one "dot" per nucleus.

The present study, which included only malignant lesions, has shown increased numbers of AgNORs per nu-

TABLE V. Carcinoma of the Breast: Relationship Between Independent Variables Including AgNORs and 4-Year Survival

Variable	Dead (n = 21)	Alive (n = 69)	Significance
Cell type:			
Small	1	12 }	
Medium	5	23 }	NS
Large	15	34 }	
Histologic grade	2.95 ± 0.22	2.88 ± 0.32	NS
NOR number	3.71 ± 1.01	3.07 ± 0.99	$t = 2.59$ df = 88 $p = 0.011$
No. of lymph nodes involved	2.76 ± 2.95	1.28 ± 1.37	$t = 2.24$ df = 22.6 $p = 0.035$
Metastasis: Negative	4	46	x2 = 12.92
Positive	17	23	df = 1 $p = 0.0003$

NS: not significant.
t: student's t test.
df: degrees of freedom.
x2: contingency tables.

cleus in high grade neoplasms when compared to low grade tumors. Large cell carcinomas in this study proved to be high grade neoplasms with aggressive behavior. More AgNORs were observed in the large cell carcinomas ($P < 0.0001$) when compared to the medium and small cells. Therefore, a significant relationship between increasing AgNOR counts and an increase in cell size and histological grade ($P < 0.0001$) was seen. Counting of these AgNORs, therefore, in lesions of the breast not only helps to distinguish benign from malignant lesions [5] but may also suggest the histological grade.

Studies have also indicated prediction of metastasis by NOR counts [6]. Gillin et al. [6] and Moran et al. [3] have predicted metastases in prostatic and colorectal cancers by doing AgNOR counts. Such a prediction could not be made in the present study, although it was seen that tumors with distant metastasis had more AgNORs (3–4) in their nucleus ($P < 0.0001$). Moran et al. [3], in their study on colorectal carcinoma on 51 patients, found that survivors had significantly lower AgNOR counts of 8–15 in primary tumors and lymph node metastasis than nonsurvivors whose counts were between 13–25 in the primary and metastatic lesions ($P < 0.05$). In their regression analysis model, NORs were the most important individual variable for predicting survival when compared to ploidy values.

This study has also proved that the number of AgNORs per nucleus was significantly associated with a 4-year survival: the higher the number of AgNORs per nucleus, the lesser the chances of surviving for 4 years. Other factors that influenced survival in this study were the number of positive lymph nodes and metastasis.

CONCLUSIONS

AgNORs prove to be a simple and easy method of assessing the type of cell involved, large, medium, or small, the histological grade of the neoplasm, and the overall behavior with regard to its metastasis. The main disadvantage of the method as found in this study was a heavy silver precipitate on the sections hindering easy counting of the AgNORs. When survival patterns were analyzed by the univariate assessment, it was found that AgNOR grade was an important variable in predicting survival for 4 years; a higher AgNOR count was significantly associated with a poorer chance of survival at the end of 4 years.

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